Effect of Glutamine Analogs on Glutaminase Formation in Pseudomonas aeruginosa

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The bacterial distribution of glutaminase activities was investigated with the cell-free extracts. Glutaminase activity was found in all the strains tested. The cell-free extract of *Pseudomonas aeruginosa* possesses the highest activity. The production of glutaminase was examined by adding glutamine analogs to the medium. The substrates and products of glutaminase reaction stimulated the production of glutaminase. L-Glutamine and L-asparagine were the most effective inducers.

INTRODUCTION

Glutaminases are ubiquitous in various organisms. The enzymes catalyze mainly the hydrolytic deamidation of L-glutamine to form L-glutamate and ammonia. Some glutaminases also catalyze the hydroxylaminolysis of glutamine in the presence of hydroxylamine to yield γ-glutamylhydroxamic acid. The presence of multiple forms of glutaminase was found in several organs of rat (1–3) and hog (4–7). Recently, it was reported that *Escherichia coli* contains two glutaminase isozymes which are easily distinguished by differences in pH optima (8–9). One isozyme has a pH optimum at 5, while the other is active above pH 7. Glutaminase of *Pseudomonas aeruginosa* was separated into two fractions on DEAE-cellulose column chromatography. These fractions were distinguished by differences in substrate specificity: glutaminase A acts on asparagine in addition to glutamine, and theanine (γ-glutamyl ethylamide) and γ-glutamylhydrazide are hydrolyzed by glutaminase B (10).

The present paper describes the bacterial distribution of glutaminase activity at physiological pH, and the effect of amino acid analogs on the production of glutaminase.

EXPERIMENTAL

Materials. L-Theanine, D-glutamine were kindly supplied from Ajinomoto Co., Tokyo. DL-β-Aspartylhydroxamic acid and L-γ-glutamylhydroxamic acid were purchased from Sigma St. Louis, Mo., U. S. A. Both enantiomers of γ-glutamylhydrazide (11), γ-glutamylmethylester (11) and DL-α-aminoadipic acid (12) were prepared according to the methods given in the literature. The other chemicals were obtained commercially.

Microorganisms. Seven strains of bacteria were used: *Escherichia coli* IFO 3208,

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3301, *Serratia marcescens* IFO 3046, *Bacillus cereus* IFO 3001, *Agrobacterium tumefaciens* IAM B–26–1, *Pseudomonas aeruginosa* IFO 3080 and *Pseudomonas fragi* IFO 3458.

Preparation of Cell-Free Extracts. The cells harvested by centrifugation were washed twice with 0.85% NaCl solution. The washed cells were suspended in 0.01 M potassium phosphate buffer, pH 7.2 and were subjected to sonication in a 19 kc Kaijo Denki oscillator at 0–7°C for 5 min. The intact cells and debris were removed by centrifugation at 17,000 × g at 0°C for 20 min. The supernatant solution was dialyzed against 0.01 M potassium phosphate buffer, pH 7.2 and employed as a cell-free extracts.

Assay Procedure. The enzymatic hydroxylaminolysis of glutamine, asparagine, γ-glutamylhydrazide, or theanine was assayed by determining hydroxamic acid formed. The standard reaction mixture was composed of 25 μmoles of L-glutamine, 300 μmoles of neutralized hydroxylamine, 40 μmoles of Tris-HCl buffer (pH 7.2) and a cell-free extract in a final volume of 1.0 ml. After incubation at 30°C for 5 to 30 min, the hydroxamic acid formed was determined as follows. The ferric reagent was prepared by mixing the same volume of 0.5 M $FeCl_3$, 0.6 M trichloroacetic acid and 2.0 N HCl (13). The ferric reagent (1.5 ml) was added to 1 ml of the reaction mixture, if necessary, precipitate was centrifuged off, and then the color intensity was measured at 540 nm. One tenth of absorbance corresponds to 0.339 μmole of L–γ-glutamylhydroxamic acid. Protein was determined by the method of Lowry *et al.* (14) with crystalline egg albumin as a standard.

Definition of Unit and Specific Activity. One unit of enzyme is the amount that catalyzes conversion of 1.0 μmole of a substrate to hydroxamic acid per min under the standard conditions. Specific activity is defined as units per mg protein.

Spectrophotometry. The absorbance was measured with a Carl Zeiss PMQ II spectrophotometer.

RESULTS

1. Distribution of Glutaminase Activity

Bacteria were grown in a medium composed of sodium glutamate 10 g, peptone 1 g, KH_2PO_4 1 g, K_2HPO_4 2 g and $MgSO_4 \cdot 7H_2O$ 0.1 g per liter of tap water (pH 7.2). The cultures were carried out with 500-ml flasks at 27°C for 24 hr under aeration. Glutaminase activities were examined with the cell-free extracts. L-Glutamine, L-asparagine and L-theanine were used substrates. Hydroxylaminolysis of these substrates was determined at pH 7.2. Table I shows that all strains of *Pseudomonas* tested possess the highest activity of glutaminase. In addition, the activities for L–asparagine and L-theanine were also the highest in the strains of *Pseudomonas*. *Pseudomonas aeruginosa* IFO 3080 was selected to study glutaminase because of having the highest activity.

2. Effect of Culture Conditions on Glutaminase Activity

Pseudomonas aeruginosa was grown at 27°C for 18 hr in a growth medium: glucose 10 g, ammonium sulfate 10 g, Na_2HPO_4 7 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $MnCl_2 \cdot 4H_2O$ 0.01 g, $CaCl_2$ 0.01 g, and $FeCl_3 \cdot 7H_2O$ 0.005 g per liter of tap water (pH 7.2). Then, amino acids were added to the medium, the incubation was continued for another 2 hr. Activities of hydroxylaminolysis of both enantiomers of glutamine, asparagine and γ-glutamylhydrazide were determined with the cell free extracts (Table II). L-Glutamine and L-asparagine enhanced the activity of hydroxylaminolysis of L-glutamine. In addition, the products of L-glutaminase and L-asparaginase, L-glutamic acid and L-aspartic acid also enhance the activity of hydroxylaminolysis of L-glutamine. D-Amino acids such as D-aspartic acid, D-glutamic

Table I. *Distribution of the Enzyme*

Strains			L-Glutamine		L-Asparagine		L-Theanine	
			S. A. (10^{-3})	T. U. (10^{-3})	S. A. (10^{-3})	T. U. (10^{-3})	S. A. (10^{-3})	T. U. (10^{-3})
Escherichia coli	IFO	3208	3.3	24.2	7.9	57.8	1.9	13.8
Escerichia coli	IFO	3301	2.6	59.9	5.3	123.4	1.4	33.1
Serratis marcescens	IFO	3046	6.2	192.3	0.2	5.0	3.5	107.9
Bacillus cereus	IFO	3001	1.6	11.6	0.5	3.5	0.7	5.0
Agrobacterium tumefaciens	IAM	B-26-1	0.8	13.3	0.8	13.3	0.7	11.2
Pseudomonas fragi	IFO	3458	96.2	2193	48.1	1097	9.3	212.0
Pseudomonas aeruginosa	IFO	3080	98.0	2528	55.7	1435	12.9	333.7

S. A. : specific activity
T. U. : total unit

Table II. *Effect of Amino Acids on Glutaminase Activity*

Amino acid (10^{-2} M)	Specific Activity					
	L-Glu-NH_2	D-Glu-NH_2	L-Asp-NH_2	D-Asp-NH_2	L-Glu-$NHNH_2$	D-Glu-$NHNH_2$
None	0.106	0.090	0.022	0.035	0.005	0.003
L-Asp	0.269	0.235	0.038	0.103	0.012	0.005
L-Glu	0.272	0.240	0.036	0.100	0.009	0.005
L-Asp-NH_2	0.344	0.290	0.046	0.112	0.009	0.005
L-Glu-NH_2	0.379	0.316	0.050	0.121	0.009	0.005
L-γ-Me-Glu	0.229	0.212	0.040	0.084	0.010	0.005
D-Asp	0.204	0.150	0.032	0.065	0.007	0.004
D-Glu	0.188	0.185	0.035	0.080	0.008	0.004
D-Asp-NH_2	0.121	0.122	0.028	0.051	0.006	0.003
D-Glu-NH_2	0.172	0.167	0.033	0.073	0.008	0.004
D-γ-Me-Glu	0.189	0.160	0.033	0.074	0.008	0.004
DL-α-AA	0.115	0.095	0.023	0.038	0.005	0.003

Abbreviations are as follows. Asp, aspartic acid; Glu, glutamic acid; Asp-NH_2, asparagine; Glu-NH_2, glutamine; γ-Me-Glu, γ-glutamyl methylester; α-AA, α-aminoadipic acid.

acid and D-glutamine also were good stimulators, but D-asparagine was not effective. The activity of L-asparagine, D-asparagine and D-glutamine hydroxylaminolyses was enhanced in a similar manner to that of L-glutamine hydroxylaminolysis. Since these amino acids are substrates for glutaminase A, this finding suggests that glutaminase A is inducibly formed. The hydroxylaminolysis activity of D- and L-γ-glutamylhydrazide, which is attributed to glutaminase B was stimulated only slightly. In any cases, D-asparagine and DL-α-aminoadipic acid were not effective to enhance the activity.

DISCUSSION

Glutaminase catalyzes the hydrolysis of amide bond of glutamine. In addition, γ-glutamyl moiety of the substrates is transferred to hydroxylamine by some glutaminases. We reported that two glutaminases (A and B) from *Pseudomonas aeruginosa* catalyze both the hydrolysis and the hydroxylaminolysis of glutamine (10). Glutaminase from *Escherichia coli* (optimum pH; 5.5) also catalyzes the formation of γ-glutamylhydroxamic acid from glutamine and the formation of γ-glutamyl methylester in the presence of methanol as well (8). γ-Glutamyltransferase from *Agaricaceae* also catalyzes the hydroxylaminolysis and the hydrolysis of glutamine (15). Asparaginases from *Escherichia coli* (16) and *Alcaligenes faecalis* (17) catalyze the hydrolysis and the hydroxylaminolysis of asparagine. These reactions by glutaminase and asparaginase have been formulated as proceeding through an acyl enzyme intermediate analogous to those found in the reactions by proteolytic enzymes, *e.g.*, chymotrypsin (18) and papain (19) as follows.

Acylation

$$\text{Glutamine} + \text{Enzyme} \rightleftharpoons \text{Glutamine}\cdots\text{Enzyme} \rightarrow \gamma\text{-Glutamyl}\cdot\text{Enzyme}$$

Deacylation

$$\gamma\text{-Glutamyl}\cdot\text{Enzyme} \xrightarrow{H_2O} \text{Glutamic acid}$$

$$\gamma\text{-Glutamyl}\cdot\text{Enzyme} \xrightarrow{NH_2OH} \gamma\text{-Glutamylhydroxamic acid}$$

It was reported that a phosphate-activated mammalian glutaminase (20) and glutaminase from *Pseudomonas putrefaciens* (21) do not catalyze the hydroxylaminolysis of glutamine but do the hydrolysis. In contrast, γ-glutamyltransferase from *Proteus vulgaris* catalyzes the hydroxylaminolysis of glutamine and some other transfer reactions, but does not catalyze the hydrolysis (22). The selectivity of nucleophile of these enzymes reflects probably the electrophilicity and some other properties of the active site.

Bacterial distribution of glutaminase and asparaginase was investigated by Wade *et al.*, who determined the activity of hydrolysis (23). A very high asparaginase activity was found in some strains of *Erwinia* species (1.6–5.3 i.u./mg protein). A high glutaminase activity was demonstrated in *Pseudomonas fluorescens, Serratia marcescens* and *Escherichia coli.* The hydrolysis activities of the glutaminases tested here were higher than the hydroxylaminolysis activities shown in Table I. It was reported that γ-glutamyltransferase of *Agariticaceae* catalyzes hydrolysis and hydroxylaminolysis of

theanine (15).

The formation of asparaginase of *Escherichia coli* was not increased when a complex medium or an amino acid free medium were supplemented with L-asparagine (24). Only among species of *Pseudomonas* has there been evidence of a marked inducible formation of asparaginase by asparagine or aspartic acid (25). Glutaminase of *Acinetobacter glutaminasificans* was reported to be induced by L-glutamic acid (26). L-Glutamic acid, L-glutamine and L-asparagine serve as good inducers for the formation of glutaminase of *Pseudomonas fluorescens* AG (27). It was demonstrated, however, that L-glutamic acid is converted into glutamine, which acts as a true inducer. Glutaminase A of *Pseudomonas aeruginosa* is induced not only such L-amino acids but also D-amino acids. It was reported that L- and D-amino acids act as inducers for the formation of L-alanine dehydrogenase of *Bacillus subtilis* (28) and also ε-lysine acylase of *Aspergillus oryzae* (29). In *Bacillus subtilis*, L- and D-alanine are interconvertible by alanine racemase. In a mutant deficient in alanine racemase, neither L-alanine nor any of the L-amino acid inducers can induce L-alanine dehydrogenase, whereas the enzyme is still inducible by D-alanine and the other D-amino acid inducers. Thus, the L-amino acids induce L-alanine dehydrogenase only if they can be converted into D-alanine through L-alanine.

Glutaminase A is inducibly formed by L- and D-glutamine, and some other L– and D-amino acids. This apparent lack of specificity may be explained by a lack of specificity in an inducer-binding site. Alternatively, the multiple inducers of glutaminase may be enzymically convertible to a common inducer. Glutaminase B is slightly inducible compared to glutaminase A.

REFERENCES

(1) N. Katunuma, I. Tomino, and H. Nishino, *Biochem. Biophys. Res. Commun.*, **22**, 321 (1966).
(2) N. Katunuma, A. Huzino, and I. Tomino, *Advances in Enzyme Regulation*, **5**, 55 (1967). Pergamon Press, Oxford and New York.
(3) N. Katunuma, I. Tomino, and Y. Sanada, *Biochem. Biophys. Res. Commun.*, **32**, 426 (1968).
(4) E. Kvamme, B. Tveit, and G. Svenneby, *Biochem. Biophys. Res. Commun.*, **20**, 566 (1965).
(5) E. Kvamme, *J. Mol. Biol.*, **52**, 239 (1970).
(6) E. Kvamme, B. Tveit, and G. Svenneby, *J. Biol. Chem.*, **245**, 1871 (1970).
(7) G. Svenneby, B. Tveit, and E. Kvamme, *J. Biol. Chem.*, **245**, 1878 (1970).
(8) S. C. Hartman, *J. Biol. Chem.*, **243**, 853 (1968).
(9) S. Prusiner and E. R. Stadtman, *Biochem. Biophys. Res. Commun.*, **45**, 1474 (1971).
(10) K. Soda, M. Ohshima, and T. Yamamoto, *Biochem. Biophys. Res. Commun.*, **46**, 1278 (1972).
(11) S. Akabori and K. Narita, *Nippon Kagaku Zasshi*, **74**, 69 (1953).
(12) T. P. Waalkes, W. S. Fones, and J. White, *J. Amer. Chem. Soc.*, **72**, 5760 (1950).
(13) F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, **159**, 21 (1945).
(14) O. H. Lowry, N. J. Rosebrough, A. L. Farrand, and R. I. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
(15) H. J. Giglotti and B. Levenberg, *J. Biol. Chem.*, **329**, 2274 (1964).
(16) N. Nakamura, Y. Morikawa, and M. Tanaka, *Agr. Biol. Chem.*, **35**, 743 (1971).
(17) H. Sakato and K. Soda, *Bull. Inst. Chem. Res., Kyoto Univ.*, **48**, 283 (1971).
(18) M. L. Bender, G. E. Clement, C. R. Gunter, and F. J. Kezdy, *J. Amer. Chem. Soc.*, **86**, 3697 (1964).
(19) L. J. Brubacher and M. L. Bender, *J. Amer. Chem. Soc.*, **88**, 5871 (1966).

(20) C. Lamer, Jr., *Biochem. Biophys. Acta.*, **151**, 188 (1968).
(21) J. S. Holcenberg, J. Roberts, and W. C. Dolowy, "The Enzymes of Glutamine Metabolism". Edited by S. Prusiner and E. R. Stadtman, Academic Press, New York and London, (1973), p. 277.
(22) H. Waelsch, "Methods in Enzymology", Vol. 2, 1955, p. 267.
(23) H. E. Wade, H. K. Robinson, and B. W. Phillips, *J. Gen. Microbiol.*, **69**, 299 (1971).
(24) J. Robert, G. Burson, and J. M. Hill, *J. Bacteriol.*, **95**, 2117 (1968).
(25) S. R. Mardashev, V. V. Eremenko, and A. Ya. Nikolaev, *Mikrobiologiya*, **39**, 11 (1970).
(26) J. Roberts, J. S. Holcenberg, and W. C. Dolowy, *J. Biol. Chem.*, **247**, 84 (1972).
(27) A. Ya. Nikolaev, N. N. Sokolov, and S. R. Mardashev, *Biokhimiya*, **36**, 643 (1970).
(28) R. Berberich, M. Kaback, and E. Freese, *J. Biol. Chem.*, **243**, 1006 (1968).
(29) I. Chibata, T. Ishikawa, and T. Tosa, *Bull. Agr. Chem. Soc. Japan*, **24**, 37 (1960).